Core E – Analysis of Neutral Lipids from Human Plasma
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This protocol describes the extraction and direct measurement of cholesterol esters (CEs) and triacylglycerols (TAGs) that are found in human plasma. Diacyl glycerols (DAGs) are extracted and then determined after derivatization with 2,4-difluorophenyl isocyanate. The steps include addition of an internal standard (IS) to separate aliquots of the thawed sample, liquid/liquid extraction, and analysis of extracts by normal phase HPLC/MS/MS using multiple reaction monitoring (MRM) for CEs, full spectral scanning (Q1) for TAGs and, after derivatization, constant neutral loss scanning (NL) for both 1,2- and 1,3-DAGs. Human plasma samples are received in screw-cap plastic vials, which are frozen on dry ice. Upon receipt, samples are stored as received at -70 ^{o}C until the neutral lipids are extracted.

Reagents required:

Dichloromethane (DCM)
Ethyl acetate
Isooctane
Methyl *tert*-butyl ether (MTBE)
Acetonitrile
Deionized water
Ammonium acetate
Dulbecco's Phosphate buffered solution (1X concentration) without calcium or magnesium (DPBS)
4-Dimethylamino pyridine (DMAP)
2,4-Difluorophenyl isocyanate (DFPI)
Appropriate Internal and Reference Standards - see table of Sample Preparation Details and descriptions of quantitative procedures below.

I. Sample Preparation - Extraction and Derivatization

All glassware is solvent rinsed with DCM and ethyl acetate prior to use.
Plasma sample preparation details are dependent on the neutral lipid species to be determined.

Sample Preparation Details

Neutral Lipid	Aliquot Volume (Step 2)	Internal Standard (IS) (Step 4)	Avanti ID	Concentration IS Solution (Step 4)	Volume IS Added (Step 4)	Final Volume (Step 10)
CE	5 μL	$^{13}C_{18}$18:1-CE (See note) 18:1-d_5-CE (Alternate)	 LM-4200	7.6 μg/μL 7.6 μg/μL	10 μL 10 μL	5 mL
TAG	1 μL	14:0/16:1/14:0-d_5-TAG	110541	200 ng/μL	10 μL	5 mL
DAG	5 μL	1,3-20:0/20:0-d_5-DAG	110540	4.0 pg/μL	25 μL	75 μL

(Note: This internal standard was synthesized according to the procedure described in PM Hutchins, RM Barkley, RC Murphy, J. Lipid Res. 2008, 49:804–813.)

1. Just prior to extraction the samples are removed from the -70 ^{o}C freezer and thawed at room temperature.
2. After a sample is thawed and equilibrated to room temperature, an aliquot sufficient for analysis of the most abundant neutral lipids is measured (see Table for volume) into a solvent-rinsed, Teflon lined screw-cap glass culture tube.
3. 2.0 mL of DPBS is added to the thawed sample.
4. Internal standards, diluted with DCM, are added to each sample (see Table for concentrations and volumes).
5. To each sample is added 2 mL of freshly prepared 25:75 (v/v) ethyl acetate:isooctane.
6. The sample is sealed, shaken, and mixed using a vortex mixer.
7. The sample is centrifuged at 3,000 RPM for 3 min at 20 ^{o}C.
8. The organic (upper) layer is transferred to a clean glass culture tube using a clean disposable glass pipette.
9. Steps 5 through 8 are repeated once and extracts are combined.

10. The extract is reduced to dryness, re-dissolved with 4% MTBE in isooctane to the final volume given in the table, sealed with a Teflon-lined cap and stored at -20 °C, or is processed immediately for the final stage of analysis.
11. Prior to CE and TAG analysis, a 50 μL aliquot is transferred to a 0.9 mL conical LC vial, sealed with a Teflon-lined crimp cap and analyzed according to the appropriate procedure in **II. A.** or **B.**
12. Prior to DAG analysis, the extract from step 10 is treated by addition of 400 μL DCM, 10 μL of a 10 μg/μL solution of DFPI in DCM, and 10 μL of a 10 μg/μL solution of DMAP in DCM.
13. The culture tube is re-sealed with a Teflon-lined cap and place in a heating block at 60 °C for 30 min.
14. The tube is removed from the heating block and stored overnight at room temperature.
15. The solution is transferred to a 0.9 mL LC vial.
16. The solution in the LC vial is taken to dryness under a gentle stream of N_2 at room temperature.
17. The extract is re-dissolved in 20 μL of 4% MTBE in isooctane and analyzed according to the procedure in **II. C.**

II. Analysis by HPLC/MS and HPLC/MS/MS

Liquid Chromatography

Individual chromatographic separations for all species of a given class of neutral lipids were performed using the following conditions with a Shimadzu HPLC consisting of a Sil-HTc controller and auto-sampler, and three LC10AD pumps:

Normal Phase Separation

Phenomenex silica column (Luna): 150 mm X 0.2 mm id, 5 μm particle size

Solvent system

Pump A-100% isooctane

Pump B-50:50 MTBE:isooctane

Gradient

Total flow – 0.2 mL/min

Time (min)	%B
0.0	Start
8.0	5.0
25.0	30.0
29.0	90.0
35.0	90.0
40.0	5.0
50.0	Stop

Auto Sampler

Injection volume –	1.0 μL
Rinse volume –	500 μL
Needle stroke –	52 mm
Rinse speed –	35 μL/s
Sampling speed –	15.0 μL
Cooler enabled –	Yes
Cooler temperature –	15 °C

Post Column Modifier

Pump C Flow rate – 0.03 mL/min

Modifier – 10 mM ammonium acetate in 5:95 (v/v) water:acetonitrile.

Mass Spectrometry

The total effluent from the HPLC was admitted to the mass spectrometer. Electrospray ionization mass spectral analyses were conducted using an Applied Biosystems 4000 QTrap LC/MS/MS system equipped with a Turbo Ion Spray source. The conditions for each class of neutral lipid are given in the descriptions and tables below.

A. Quantitation of Cholesterol Esters

Cholesterol esters are quantified from multiple reaction monitoring (MRM) data and application of standard curves that relate the responses for known amounts of reference standards 18:1-, 18:2- and 20:4-CE to that for a single internal standard. The curves are prepared from solutions of reference standards at 0, 0.29, 0.98, 2.9, and 9.8 μg/5mL that also contain the same amount of IS that is used for

extracts of plasma. For species for which there is no standard curve, the standard curve used for quantitation is that for the closest reference standard based on degree of unsaturation and composition of the fatty acid side chain. Some species in the table will be present at levels below the limit of quantitation.

MRM Conditions		Mass Spectrometer Parameters	
Polarity –	Positive	CUR –	10.0
Scan mode –	MRM	IS –	5500.0
Ion source –	Turbo Spray	TEM –	300.0
Resolution Q1 –	Unit	GS1 –	40.0
Resolution Q3 –	Unit	GS2 –	20.0
Intensity Threshold –	0.00 cps	ihe –	ON
Settling Time –	0.000 ms	CAD –	7.0
MR pause –	5.0070 ms	DP –	60.0
MCA –	No	EP –	9.0
Step Size –	0.00 amu	CE –	25.0
Cycle Time –	2.63 s	CXP –	6.0
Transitions –	See table below		

Cholesterol Ester MRM Transitions

LM ID	Cholesterol Ester (Acyl Carbon Number: Double bonds)	Molecular Mass	Q1 $[NH_4]^+$	Q3	Dwell Time (ms)
LMST01020021	14:1	594.6	612.6	369.3	100
LMST01020004	14:0	596.6	614.6	369.3	100
LMST01020022	15:1	608.6	626.6	369.3	100
LMST01020027	15:0	610.6	628.6	369.3	100
LMST01020024	16:2	620.6	638.6	369.3	100
LMST01020006	16:1	622.6	640.6	369.3	100
LMST01020005	16:0	624.6	642.6	369.3	100
LMST01020023	17:1	636.6	654.6	369.3	100
LMST01020026	17:0	638.6	656.6	369.3	100
LMST01020009	18:3	646.6	664.6	369.3	100
LMST01020008	18:2	648.6	666.6	369.3	100
LMST01020003	18:1	650.6	668.6	369.3	100
LMST01020007	18:0	652.7	670.7	369.3	100
LMST01020030	18:1-d_5 IS (Alternate)	655.7	673.7	369.3	100
	$^{13}C_{18}$-18:1 IS	668.7	686.7	369.3	100
LMST01020014	20:4	672.6	690.6	369.3	100
LMST01020013	20:3	674.6	692.6	369.3	100
LMST01020012	20:2	676.7	694.7	369.3	100
LMST01020011	20:1	678.7	696.7	369.3	100
LMST01020010	20:0	680.7	698.7	369.3	100
LMST01020019	22:6	696.6	714.6	369.3	100
LMST01020031	22:5	698.6	716.6	369.3	100
LMST01020018	22:4	700.6	718.6	369.3	100
LMST01020017	22:2	704.7	722.7	369.3	100
LMST01020025	22:1	706.7	724.7	369.3	100
LMST01020016	22:0	708.7	726.7	369.3	100

B. Quantitation of Triacylglycerols

Triacylglycerols are quantified by integration of molecular ions (as ammonium ion adducts) from full scan mass spectra and application of standard curves that relate the responses of known amounts of reference standards to that for a single internal standard. The curves are prepared from solutions of reference standards at 0, 5, 10, 25, 50, 100, 200, and 400 ng/5mL that also contain the same amount of IS that is used for extracts of plasma. The reference standards used are 49:2- (Avanti ID 110528), 50:1- (Avanti ID 110521), 50:0- (Avanti ID 110520) and 54:3- (Avanti LM3216) TAGs. For species for which there is no standard curve, the standard curve used for quantitation is that for the closest reference standard based on degree of unsaturation and composition of the fatty acid side chains. Final calculations of amounts of analyte per sample require isotope corrections and application of deisotope factors. Some species in the table will be present at levels below the limit of quantitation.

Q1 Scan Conditions

Polarity –	Positive
Scan mode –	Profile
Ion source –	Turbo Spray
Resolution Q1 –	Unit
Intensity threshold –	0.00 cps
Settling Time –	0.00 ms
MR pause –	5.0070 ms
MCA –	No
Center/Width –	No
Step size –	0.1 amu
Scan range –	500-1200 amu
Scan time –	4 s

Mass Spectrometer Parameters

Cur –	14.0
IS –	5500.0
TEM –	300.0
GS1 –	1800
GS2 –	20.0
ihe –	ON
DP –	88.0
EP –	10.0

Triacylglycerol Ions

TAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$	TAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$
40:2	$C_{43}H_{78}O_6$	690.6	708.6	44:0	$C_{47}H_{90}O_6$	750.7	768.7
40:1	$C_{43}H_{80}O_6$	692.6	710.6	IS	$C_{47}{}^2H_5H_{83}O_6$	753.7	771.7
40:0	$C_{43}H_{82}O_6$	694.6	712.6	45:3	$C_{48}H_{86}O_6$	756.6	774.7
41:5	$C_{44}H_{74}O_6$	698.6	716.6	45:2	$C_{48}H_{88}O_6$	760.7	778.7
41:4	$C_{44}H_{76}O_6$	700.6	718.6	45:1	$C_{48}H_{90}O_6$	762.7	780.7
41:3	$C_{44}H_{78}O_6$	702.6	720.6	45:0	$C_{48}H_{92}O_6$	764.7	782.7
41:2	$C_{44}H_{80}O_6$	704.6	722.6	46:5	$C_{49}H_{84}O_6$	768.6	786.7
41:1	$C_{44}H_{82}O_6$	706.6	724.6	46:4	$C_{49}H_{86}O_6$	770.6	788.7
41:0	$C_{44}H_{84}O_6$	708.6	726.7	46:3	$C_{49}H_{88}O_6$	772.7	790.7
42:5	$C_{45}H_{76}O_6$	712.6	730.6	46:2	$C_{49}H_{90}O_6$	774.7	792.7
42:4	$C_{45}H_{78}O_6$	714.6	732.6	46:1	$C_{49}H_{92}O_6$	776.7	794.7
42:3	$C_{45}H_{80}O_6$	716.6	734.6	46:0	$C_{49}H_{94}O_6$	778.7	796.7
42:2	$C_{45}H_{82}O_6$	718.6	736.7	47:5	$C_{50}H_{86}O_6$	782.6	800.7
42:1	$C_{45}H_{84}O_6$	720.6	735.7	47:4	$C_{50}H_{88}O_6$	784.7	802.7
42:0	$C_{45}H_{86}O_6$	722.6	740.7	47:3	$C_{50}H_{90}O_6$	786.7	804.7
43:2	$C_{46}H_{84}O_6$	732.6	750.7	47:2	$C_{50}H_{92}O_6$	788.7	806.7
43:1	$C_{46}H_{86}O_6$	734.6	752.7	47:1	$C_{50}H_{94}O_6$	790.7	808.7
43:0	$C_{46}H_{88}O_6$	736.7	754.7	47:0	$C_{50}H_{96}O_6$	792.7	810.7
44:5	$C_{47}H_{80}O_6$	740.6	758.6	48:6	$C_{51}H_{86}O_6$	794.6	812.7
44:4	$C_{47}H_{82}O_6$	742.6	760.6	48:5	$C_{51}H_{88}O_6$	796.7	814.7
44:3	$C_{47}H_{84}O_6$	744.6	762.7	48:4	$C_{51}H_{90}O_6$	798.7	816.7
44:2	$C_{47}H_{86}O_6$	746.6	764.7	48:3	$C_{51}H_{92}O_6$	800.7	818.7
44:1	$C_{47}H_{88}O_6$	748.7	766.7	48:2	$C_{51}H_{94}O_6$	802.7	820.7

TAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4]^+$
48:1	$C_{51}H_{96}O_6$	804.7	822.8
48:0	$C_{51}H_{98}O_6$	806.7	824.8
49:5	$C_{52}H_{90}O_6$	810.7	828.7
49:4	$C_{52}H_{92}O_6$	812.7	830.7
49:3	$C_{52}H_{94}O_6$	814.7	832.7
49:2	$C_{52}H_{96}O_6$	816.7	834.7
49:1	$C_{52}H_{98}O_6$	818.7	836.8
49:0	$C_{52}H_{100}O_6$	820.8	838.8
50:5	$C_{53}H_{92}O_6$	824.7	842.7
50:4	$C_{53}H_{94}O_6$	826.7	844.7
50:3	$C_{53}H_{96}O_6$	828.7	846.8
50:2	$C_{53}H_{98}O_6$	830.7	848.8
50:1	$C_{53}H_{100}O_6$	832.8	850.8
50:0	$C_{53}H_{102}O_6$	834.8	852.8
51:5	$C_{54}H_{94}O_6$	838.7	856.7
51:4	$C_{54}H_{96}O_6$	840.7	858.8
51:3	$C_{54}H_{98}O_6$	842.7	860.8
51:2	$C_{54}H_{100}O_6$	844.8	864.8
51:1	$C_{54}H_{102}O_6$	846.8	864.8
51:0	$C_{54}H_{104}O_6$	848.8	866.8
52:5	$C_{55}H_{96}O_6$	852.7	870.8
52:4	$C_{55}H_{98}O_6$	854.7	872.8
52:3	$C_{55}H_{100}O_6$	856.8	874.8
52:2	$C_{55}H_{102}O_6$	858.8	876.8
52:1	$C_{55}H_{104}O_6$	860.8	878.8
52:0	$C_{55}H_{106}O_6$	862.8	880.8
53:5	$C_{56}H_{98}O_6$	866.7	884.8
53:4	$C_{56}H_{100}O_6$	868.8	886.8
53:3	$C_{56}H_{102}O_6$	870.8	888.8
53:2	$C_{56}H_{104}O_6$	872.8	890.8
53:1	$C_{56}H_{106}O_6$	874.8	892.8
53:0	$C_{56}H_{108}O_6$	876.8	894.8
54:6	$C_{57}H_{98}O_6$	878.7	896.8
54:5	$C_{57}H_{100}O_6$	880.8	898.8
54:4	$C_{57}H_{102}O_6$	882.8	900.8
54:3	$C_{57}H_{104}O_6$	884.8	902.8
54:2	$C_{57}H_{106}O_6$	886.8	904.8
54:1	$C_{57}H_{108}O_6$	888.8	906.8
54:0	$C_{57}H_{110}O_6$	890.8	908.8
55:6	$C_{58}H_{100}O_6$	892.8	910.8
55:5	$C_{58}H_{102}O_6$	894.8	912.8
55:4	$C_{58}H_{104}O_6$	896.8	914.8
55:3	$C_{58}H_{106}O_6$	898.8	916.8
55:2	$C_{58}H_{108}O_6$	900.8	918.8
55:1	$C_{58}H_{110}O_6$	902.8	920.9
55:0	$C_{58}H_{112}O_6$	904.9	922.9
56:6	$C_{59}H_{102}O_6$	906.8	924.8
56:5	$C_{59}H_{104}O_6$	908.8	926.8
56:4	$C_{59}H_{106}O_6$	910.8	928.8
56:3	$C_{59}H_{108}O_6$	912.8	930.8
56:2	$C_{59}H_{110}O_6$	914.8	932.9
56:1	$C_{59}H_{112}O_6$	916.8	934.9
56:0	$C_{59}H_{114}O_6$	918.9	936.9
57:6	$C_{60}H_{104}O_6$	920.8	938.8
57:5	$C_{60}H_{106}O_6$	922.8	940.8
57:4	$C_{60}H_{108}O_6$	924.8	942.8
57:3	$C_{60}H_{110}O_6$	926.8	944.9
57:2	$C_{60}H_{112}O_6$	928.9	946.9
57:1	$C_{60}H_{114}O_6$	930.9	948.9
57:0	$C_{60}H_{116}O_6$	932.9	950.9
58:6	$C_{61}H_{106}O_6$	934.8	952.8
58:5	$C_{61}H_{108}O_6$	936.8	954.8
58:4	$C_{61}H_{110}O_6$	938.8	956.9
58:3	$C_{61}H_{112}O_6$	940.9	958.9
58:2	$C_{61}H_{114}O_6$	942.9	960.9
58:1	$C_{61}H_{116}O_6$	944.9	962.9
58:0	$C_{61}H_{118}O_6$	946.9	964.9
60:12	$C_{63}H_{98}O_6$	950.7	968.8
59:2	$C_{62}H_{116}O_6$	956.9	974.9
59:1	$C_{62}H_{118}O_6$	958.9	976.9
59:0	$C_{62}H_{120}O_6$	960.9	978.9
60:4	$C_{63}H_{114}O_6$	966.9	984.9
60:3	$C_{63}H_{116}O_6$	968.9	986.9
60:2	$C_{63}H_{118}O_6$	970.9	988.9
60:1	$C_{63}H_{120}O_6$	972.9	990.9
60:0	$C_{63}H_{122}O_6$	974.9	992.9

C. Quantitation of Diacylglycerols

Diacyglycerols are quantified by integration of molecular ions (as ammonium ion adducts) from constant neutral loss mass spectra and application of standard curves that relate the responses of known amounts of reference standards to that for a single internal standard for both the 1,2- and 1,3-DAG species. The curves for quantitation of 1,2- and 1,3-DAGs was prepared from solutions of 1,2-16:0/18:0-DAG at 0, 1.2, 6.2, 12.4, 31, 62, 124, and 248 pg/75µL and 1,3-18:0/18:0 DAG at 0, 1.1, 5.6, 11.2, 28, 56, 112, and 224 pg/75µL that also contain the same amount of IS that is used for extracts of plasma. Final calculations of amounts of analyte per sample require isotope corrections and application of deisotope factors. Some species in the table will be present at levels below the limit of quantitation.

Constant Neutral Loss Conditions

Polarity –	Positive
Scan mode –	Profile
Ion source –	Turbo Spray
Neutral loss –	190.1 Da
Resolution Q1 –	Unit
Resolution of Q3	Unit
Intensity threshold –	0.00 cps
Settling Time –	5.000 ms
MR pause –	5.0070 ms
MCA –	No
Center/Width –	No
Step size –	0.1 amu
Scan range –	500-1200 amu
Scan time –	5 s

Mass Spectrometer Parameters

Cur –	10.0
IS –	5500.0
TEM –	300.0
GS1 –	40.0
GS2 –	20.0
ihe –	ON
CAD –	7.0
DP –	85.0
EP –	9.0
CE –	52.0
CXP –	6.0

Diacylglycerol Ions

DAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4\text{-DFPI}]^+$	DAG (CN:DB)	Formula	Molecular Mass	Mass $[NH_4\text{-DFPI}]^+$
30:2	$C_{33}H_{60}O_5$	536.4	709.5	36:2	$C_{39}H_{72}O_5$	620.5	793.6
30:1	$C_{33}H_{62}O_5$	538.5	711.5	36:1	$C_{39}H_{74}O_5$	622.6	795.6
30:0	$C_{33}H_{64}O_5$	540.5	713.5	36:0	$C_{39}H_{76}O_5$	624.6	797.6
32:5	$C_{35}H_{58}O_5$	558.5	731.5	37:5	$C_{40}H_{68}O_5$	628.5	801.5
32:4	$C_{35}H_{60}O_5$	560.5	733.5	37:4	$C_{40}H_{70}O_5$	630.5	803.5
32:3	$C_{35}H_{62}O_5$	562.5	735.5	37:3	$C_{40}H_{72}O_5$	632.5	805.5
32:2	$C_{35}H_{64}O_5$	564.5	737.5	37:2	$C_{40}H_{74}O_5$	634.5	807.5
32:1	$C_{35}H_{66}O_5$	566.5	739.5	37:1	$C_{40}H_{76}O_5$	636.5	809.5
32:0	$C_{35}H_{68}O_5$	568.5	741.5	37:0	$C_{40}H_{78}O_5$	638.5	811.5
33:1	$C_{36}H_{68}O_5$	580.5	753.5	38:6	$C_{41}H_{68}O_5$	640.5	813.6
33:0	$C_{36}H_{70}O_5$	582.5	755.5	38:5	$C_{41}H_{70}O_5$	642.5	815.6
34:4	$C_{37}H_{64}O_5$	588.5	761.5	38:4	$C_{41}H_{72}O_5$	644.5	817.5
34:3	$C_{37}H_{66}O_5$	590.5	763.5	38:3	$C_{41}H_{74}O_5$	646.6	819.6
34:2	$C_{37}H_{68}O_5$	592.5	765.5	38:2	$C_{41}H_{76}O_5$	648.6	821.6
34:1	$C_{37}H_{70}O_5$	594.5	767.5	38:1	$C_{41}H_{78}O_5$	650.6	823.6
34:0	$C_{37}H_{72}O_5$	596.5	769.5	38:0	$C_{41}H_{80}O_5$	652.6	825.6
35:4	$C_{38}H_{64}O_5$	600.5	773.5	40:7	$C_{43}H_{70}O_5$	666.5	839.6
35:3	$C_{38}H_{66}O_5$	602.5	775.5	40:6	$C_{43}H_{72}O_5$	668.5	841.6
35:2	$C_{38}H_{68}O_5$	604.5	777.5	40:5	$C_{43}H_{74}O_5$	670.6	843.6
35:1	$C_{38}H_{70}O_5$	606.5	779.5	40:4	$C_{43}H_{76}O_5$	672.6	845.6
35:0	$C_{38}H_{72}O_5$	608.5	781.5	40:3	$C_{43}H_{78}O_5$	674.6	847.6
36:5	$C_{39}H_{66}O_5$	614.5	787.5	40:2	$C_{43}H_{80}O_5$	676.6	849.6
36:4	$C_{39}H_{68}O_5$	616.5	789.5	40:1	$C_{43}H_{82}O_5$	678.6	851.6
36:3	$C_{39}H_{70}O_5$	618.5	791.6	40:0	$C_{43}H_{84}O_5$	680.6	853.6
				IS	$C_{43}{}^2H_5H_{79}O_5$	685.6	858.6